Development of Ribozymes For Gene Therapy

Sean M. Sullivan
Ribozyme Pharmaceuticals, Inc., Boulder, Colorado, U.S.A.

Ribozymes are a class of RNA molecules that can perform catalytically in the absence of protein. Specifically, they can hybridize to and cleave target RNA molecules independent of cellular proteins. The cleaved target RNA can not be translated thereby preventing synthesis of a specific protein. The therapeutic application is to target the ribozyme to the mRNA of a key protein or proteins involved in maintaining a disease state resulting in a cure. The ribozymes can be chemically synthesized and delivered to cells or they can be expressed from an expression vector following either permanent or transient transfection. Therapeutic applications of ribozymes have been in the areas of AIDS and cancer. The following article describes the ribozymes in more detail with respect to optimizing the design to obtain the maximal cleavage rate, identifying cleavage sites within the target RNA and delivering the ribozymes to cells of interest both *in vitro* and *in vivo*. *J Invest Dermatol 103:85S–89S, 1994*

Ribozymes are RNA molecules that hybridize to a complementary sequence of RNA and cleave that sequence in the absence of proteins. The ribozyme is composed of two domains, a binding domain and an RNA cleaving domain. The ribozyme hybridizes to the target RNA via Watson-Crick base pairing resulting in the orientation of the cleavage sequence within the target RNA to be acted upon by the catalytic domain of the ribozyme. Only then is the ribozyme able to cleave the target RNA. The ribozyme also requires divalent cation for cleavage. After cleavage of the target RNA, the cleavage products dissociate from the ribozyme making it available to hybridize to another sequence.

Development of this technology into a therapy requires the following: i) identification of a key protein responsible for a disease state, ii) the nucleotide sequence for that protein must be known to select the ribozyme cleavage sites, iii) the ribozyme must cleave the target RNA, and iv) the ribozyme must be taken up by the cells of the diseased tissue and cleave the target RNA inside the cell.

Several examples exist in the literature using ribozymes to prevent specific proteins from being synthesized. These ribozymes were either expressed from an expression vector or formulated in a delivery vehicle to facilitate cellular uptake. An active area of applications has been in the development of AIDS theapeutics [1–4]. Ribozymes have been designed to cleave the RNA coding for the gag protein [5] the 5′ leader sequence in the LTR [6] and the protease [7]. The most effective application consists of a hairpin ribozyme directed against the 5′ leader sequence [8,9]. The ribozyme was incorporated into a retroviral vector and transfected into Jurkat cells and shown to protect these cells against HIV infection using the HIV IIIb st ain. This vector was then shown to transform primary immune cells and protect those cells from infection by several different strains of HIV-I including two clinical isolates [9]. These results spurred the preparation and submission of a human gene therapy protocol for the treatment of HIV. This protocol has been approved for phase I clinical safety trials.

There have been two reported applications in the area of cancer. The first involves inhibition of cell proliferation by a ribozyme directed against the *ras* oncogene [10–12]. There is a point mutation in the *ras* oncogene converting a GUG nucleotide sequence to a GUC. The GUG triplet can not be cleaved by a hammerhead ribozyme whereas the GUC triplet can be cleaved. The ribozyme reduced the amount of mRNA from the *ras* oncogene and inhibited cell proliferation. The second example involves the treatment of Chronic Myelogenous Leukemia (CML) [13,14]. In the first stage of lymphocyte transformation, there is a translocation of c-abl from chromosome 9 to the break point cluster region of chromosome 22 resulting in the formation of a bcr-abl fusion protein. A ribozyme cleavage site is created at the junction of these two proteins. A ribozyme was designed so that one binding arm hybridized to the *bcr* side and the other binding arm of the ribozyme hbyridized to the *abl* side of the mRNA, such that only the mRNA of the fusion product would be cleaved and not the RNA from either *bcr* or *abl*. A chemically synthesized ribozyme composed of both DNA and RNA was introduced into these cells by cationic lipids. The results showed that the ribozyme reduced the amount of the fusion protein and the fusion protein mRNA, and cell proliferation was inhibited. However, there was no effect on the amount of c-abl protein, thus demonstrating the specificity of the ribozyme [13]. A similar result was observed expressing the ribozyme from a retroviral vector [14]. The design of the ribozyme was similar to the chemically synthesized ribozyme and inhibition of cell proliferation was observed.

Application of this technology is not only limited to human therapeutics, but to other areas, such as agriculture. Specifically, a ribozyme was directed against the tobacco mosaic virus (TMV) RNA-dependent RNA polymerase, a crucial protein for replication. The ribozyme was expressed from a transient expression vector transfected into tobacco plant protoplasts. Ribozyme activity was compared to an antisense construct to the same area as the ribozyme sequence. The antisense construct reduced virus production by 20% whereas the ribozyme inhibited virus production by 90% [38]. This ribozyme construct was used to generate transgenic plants and shown to have retardation in symptom development as the result of TMV infection.

These examples show that to develop an effective ribozyme based therapy requires the identification of a key protein, which in turn requires knowledge of the mechanism for the disease. The design of effective ribozymes to cleave the mRNA of this protein is

Reprint requests to: Sean M. Sullivan, Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Way, Boulder, CO 80301.

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absolutely paramount to achieving a ribozyme-based therapy. The following describes in more detail how ribozymes work and how they can be optimized to be effective therapeutics.

RIBOZYME CHARACTERISTICS

There are five type of ribozyme motifs: group I introns, ribonuclease P, hammerhead, hairpin and hepatitis delta virus ribozymes. The group I intron motif was discovered by Dr. Tom Cech and colleagues at the University of Colorado in Boulder. The natural role of this ribozyme is to process ribosomal RNA. Splicing of the rRNA intron occurs by a two step mechanism. In the first step, guanine nucleotide is added to the 5′ end of the intron as the intron/exon junction is being cleaved. In the second step, the freed 5′ exon attacks at the 3′ intron/exon junction to release the intron and to generate a spliced exon [15,6]. Ribonuclease P was discovered in bacteria by Dr. Sydney Altman and colleagues. The ribozyme is composed of a small subunit protein and a catalytic RNA. The biologic role is to generate the mature 5′-end of tRNAs by endonucleocatalytic cleavage of precursor transcripts [17]. Dr. Robert Symons and colleagues at the CSIRO in Australia described the hammerhead motif in viroids, which are helper viruses found in plants. The RNA is synthesized in a rolling circle and the hammerhead, upon hybridization, cleaves the polycistronic message into individual RNA transcripts [18]. The hairpin ribozyme was discovered by Dr. George Bruening and colleagues at the University of California, Davis [19]. The role of the hairpin is also found in plant viroids and has a similar role as the hammerhead [20,21]. The hepatitis delta virus is a helper virus and its RNA was found to have autocatalytic RNA processing similar to the function of the hairpin and hammerhead motifs [22]. The details of this ribozyme motif are still being characterized with respect to minimal sequence and substrate requirements.

HAMMERHEAD RIBOZYME DESIGN

The hammerhead ribozyme was selected for development as a therapeutic for several reasons. It is the smallest of the ribozyme motifs and is therefore appealing for chemical synthesis. It has been the best characterized ribozyme with respect to optimized target sequence, conserved nucleotides sequences within the catalytic core, and the kinetic parameters. In addition, the target RNA cleavage sites have the least constraints with respect to sequence and therefore are the most abundant sites compared to the other motifs. **Figure 1** depicts the formed hammerhead motif from bound ribozyme and substrate. The non-base paired nucleotides flanking both sides of stem II make up the catalytic core. These sequences are the CUGAUGA on the 5′ side of stem II and the GAAA on the 3′ side of stem II. Stem II can vary in sequence and length. The combination of a high GC content in the stem and the GAAA tetra loop stabilizes this structure.

The substrate and the ribozyme hybridize to form stem I and stem III, orienting the -UH- nucleotides of the substrate over the catalytic core. The H nucleotide can be an adenine, cytosine or uridine but not a guanine. Ruffner and Uhlenbeck [23] identified a GUC nucleotide triplet to yield the highest rate of cleavage compared to the other cleavage triplet sequences. However, it has been our experience that the optimal triplet is dependent upon the ribozyme binding arm composition and the nucleotide composition of the target RNA.

The proposed mechanism of a hammerhead mediated RNA cleavage begins with deprotonation of the 2′ sugar at the 3′ side of the cleavage site. Deprotonation results in nucleophilic attack of the adjacent phosphodiester bond and subsequently protonation of the 5′ oxyanion leaving group generating 2′,3′-cyclic phosphate and 5′ hydroxyl termini. The 2′ hydroxyl adjacent to the cleavage site in the substrate is essential for cleavage. The rates of ribozyme hybridization to the substrate, dissociation of the ribozyme from the substrate, and dissociation of the cleavage products from the ribozyme are controlled by the length and nucleotide composition of the binding arms. Hence, the dependence of binding arm lengths on the cleavage rate should be determined for each ribozyme of interest. The arm lengths can be optimized using either short target substrates (≤20 nucleotides) or long substrates (≥500 nucleotides). However, optimization should also be coordinated with a biologic assay because the ultimate test is the inactivation of an RNA transcript inside the cell. Optimization of the arm lengths of an expressed ribozyme should be performed in the context of the expression vector, which would involve extracting the RNA from the transfected cell and assaying for cleavage in the presence of other cellular RNAs.

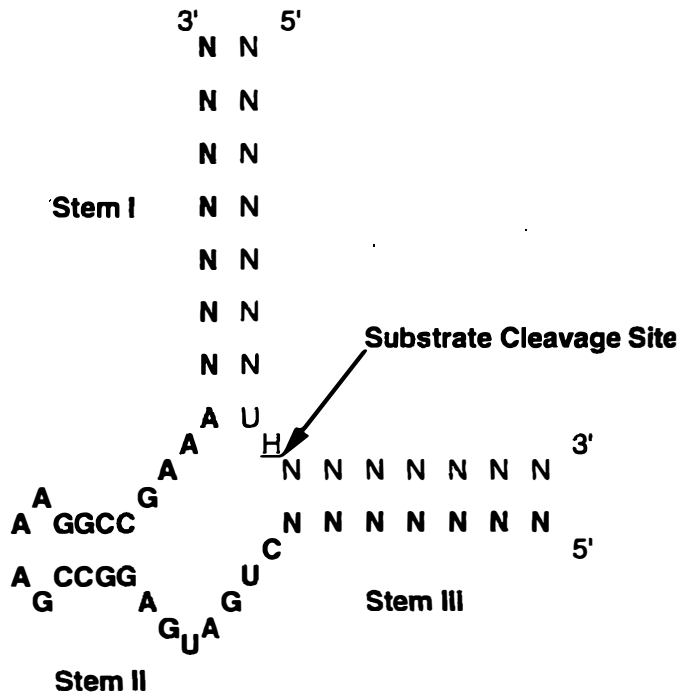

Figure 1. Structure of the hammerhead ribozyme. The hammerhead ribozyme is in bold. Stem I and stem II are the binding arms and stem II is part of the catalytic core. The H represents the cleavage site of the substrate RNA.

NUCLEASE STABILITY

Ribozymes are composed of RNA and are therefore susceptible to degradative enzymes inside and outside the cell. Development of a ribozyme that is resistant to these degradative nucleases should increase the rate of ribozyme accumulation in the cell, thereby decreasing the effective dose required for inhibition of protein synthesis. Chemical modification of the phosphate backbone and derivatization of the 2′-OH of the ribose have been strategies to achieve this goal. The phosphodiester backbone was changed to a phosphorothioate and modifications to the 2′-OH of the ribose have included 2′-fluoro [24], 2′-amino [24], 2′-O-methyl [25], 2′-O-allyl [26] and 2′-deoxy [27,28]. Some of the modifications reduced the cleavage rate of the ribozyme and, therefore, there must be a balance between increasing the nuclease stability and decreasing the ribozyme cleavage activity. Reduction in cleavage activity was dependent upon the nature of the modification and the site of insertion in the hammerhead ribozyme. A ribozyme containing three terminal phosphorothioates at the 3′ end and one terminal phosphorothioate at the 5′ end along with 2′-fluoro-2′-deoxyuridines and two 2′-amino-2′-deoxyuridines increased the $t_{1/2}$ from less than 1 min to 3 h in serum with a 40% loss in cleavage activity [2,24]. The development of a synthetic ribozyme resistant to nuclease degradation is a key factor in making this technology a viable parenteral administered pharmaceutical.

Ribozymes expressed from a DNA vector can be engineered to increase intracellular stability. This is accomplished by creating secondary structures within the RNA that inhibit nuclease degradation. Stem-loops are one type of structure and are formed by intrastrand base pairs. Placement of stem loops on either side of a

ribozyme was shown to slow the rate of ribozyme degradation and produced a greater reduction in the amount of a specific protein compared to a ribozyme lacking these structures [29]. Exonuclease degradation is initiated at either the 3′ end or the 5′ end of the RNA. Circularizing the RNA would make these ends inaccessible to the exonuclease. The effect of circular RNA on reducing nuclease degradation was tested using the hepatitis delta virus ribozyme motif. The ribozyme was expressed in such a way that processing of the transcript yielded a circular ribozyme [30]. The circularized ribozyme was shown to retain catalytic activity and serum nuclease degradation was dramatically reduced. This is a promising approach that is being evaluated for ribozyme activity inside cells.

RIBOZYME SELECTION

The rate of cleavage of a target RNA is dependent upon the accessibility of the cleavage site. Hammerhead ribozyme cleavage sites are selected by first identifying the U-H doublets in the nucleotide sequence of the target RNA with U being uridine and H being a cytosine, uridine, or adenine but not a guanine. The next round of selection is based on the degree of homology between the animal and human sequences or in the case of HIV, the most conserved sequences between the different strains of virus. The ribozyme is then folded with the corresponding sequence using a computer folding program. Only, those sites that yield a successful ribozyme motif are then tested for accessibility. Site accessibility is assayed using a combination of deoxyoligonucleotides and RNase H with *in vitro* transcripts. The deoxyoligonucleotides are hybridized to the *in vitro* transcripts in vast excess of RNase H. The enzyme degrades RNA from the DNA/RNA heteroduplex. The minimum length for the deoxyoligonucleotides has been 13 nucleotides and the ribozyme cleavage site is placed in the middle of the oligonucleotide. The RNA transcripts are uniformly labeled with ^{32}P nucleotides and the intact transcript and the two cleavage products are visualized on the gel by autoradiography. A data base has been established to correlate the accessibility results with the predicted RNA structure using a computer folding program. The validation of the computer fold of the RNA transcript to select ribozyme cleavage sites is in progress. The establishment of this data base should expedite the ribozyme development process.

Ribozymes to the identified accessible sites are synthesized and assayed for activity. The most active ribozymes are then assayed for activity in tissue culture.

RIBOZYME DELIVERY

Three options exist for ribozyme delivery: uptake of synthetic ribozymes, expression from a transient expression vector, or expression from an integrated vector. Each delivery option has its own application. Synthetic ribozymes behave very similar to conventional drugs in that they are applicable to diseases that require multiple administrations. The additional advantage being a defined mechanism of action and lack of cytotoxic side effects.

Synthetic ribozymes are taken up by cells, but the uptake efficiency is low. This efficiency can be increased through the use of delivery vehicles. Cationic lipids have been successful in facilitating delivery of the transient expression vectors [31], messenger RNA [32], and antisense DNA [33] to cells both *in vitro* and *in vivo* [34]. They are also able to facilitate ribozyme delivery (unpublished results). The cationic lipids are commercially available and the protocol is very simple. The ribozyme is added to the cationic liposomes and a complex forms spontaneously. The ribozyme/cationic lipid complex is then added to the cells. For *in vitro* use, there are two restrictions. These lipids are toxic to the cells and a toxicity profile of the cationic lipid/ribozyme complex must be established prior to efficacy studies. Secondly, the cells must be in a serum free environment during incubation with the complex. A 2–4 h incubation is sufficient for ribozyme uptake. For a more detailed discussion of optimizing activity and minimizing toxicity, see Felgner *et al* [35].

For cells that cannot tolerate the cationic lipid or lack of serum, phospholipid liposomes have been used for ribozyme delivery. Liposomes are hollow spheres composed of phospholipids arranged in a bilayer configuration and the ribozymes are trapped inside the liposomes. For a description of phospholipid liposome preparation see Sullivan, 1993 [36]. The liposomes rely on endocytosis to gain entry into the cell. Upon entry, they are degraded and the ribozymes are released into the cytoplasm. Uptake and release from endocytic vacuoles are dependent upon cell type and phospholipid composition. This is particularly true for cytoplasmic release of liposome contents. Release is dependent upon the lipid degradative enzymes in the endosome and lysosome. For example, a liposome formulation composed of phosphatidylethanolamine, phosphatidylcholine, and cholesterol was actively taken up by vero cells yielding 70% of the ribozymes in the cytoplasm. The same formulation was tested for uptake by HeLa cells showing that less than 5% of the offered dose was taken up and the ribozymes were sequestered inside intracellular vacuoles. The present state of the art requires that the lipid composition be designed for the cell type of interest and both uptake and intracellular release of the ribozyme must be taken into account.

In vivo delivery of synthetic ribozymes was achieved for topical delivery using polyacrylic acid, a controlled release polymer. The properties of the polyacrylic acid are that upon hydration, the polyacrylic acid is a liquid at pH 5. Neutralization with a weak base converts the solution to a gel. Penetration of the gel by cations collapses the gel back to a liquid. The ribozymes were formulated with polyacrylic acid and applied to mouse eyes as a liquid. An 83 pmol dose was applied and 5% was retained by the eye. The percent of ribozyme retained by the eye was linear over the range of 83 pmol to 1660 pmol. Autoradiography of ^{33}P-radiolabeled ribozyme showed localization of the ribozyme in the outer most layers of the corneal epithelium after 10 min. After 30 min the ribozyme had penetrated into the lower area of the epithelium layer and into the stromal layer. This study showed that synthetic ribozymes could be maintained at a level within tissue from a local administration. These results may be more generally applicable to other topical administration routes.

Ribozyme expression from an expression vector falls under the category of gene therapy. Delivery and expression of a ribozyme expression vector presently relies upon the use of non-replicating viruses, such as retrovirus, adenovirus or adeno-associated virus. Retroviruses and adeno-associated viruses result in stable transfectants whereas infection with adenovirus results in transient transfectants. Hence, infection with retroviruses and adeno-associated viruses yields permanent transfections that have the potential for continuous ribozyme expression and adenovirus produces a transient transfection that yields transient ribozyme expression. Safety issues surrounding the integration of foreign DNA derived from a virus are still being addressed and for this reason ribozyme gene therapy may be used for limited applications. For example, transient expression of ribozymes may be applicable to non-life threatening acute diseases, such as restenosis, where multiple administrations cannot be tolerated. Ribozyme expression from an integrated vector may be applicable to life threatening chronic diseases, such as AIDS, where multiple administrations can not be tolerated.

Vector transfection for transient expression can take two forms. The first is to use adenoviral vectors as a means for ribozyme to be introduced into the cells and expressed. The advantages of adenovirus are a high transfection efficiency, a therapeutic level of protein expression, and high titers from packaging cell lines [39]. Expression of proteins has been maintained for 1–3 weeks after *in vivo* transfection. A disadvantage of adenovirus vectors involves the potential of recombination between replication and replication competent vectors. Another concern is the immunogenicity of this virus creating immune based inflammation problems [40].

The second form is non-viral mediated DNA vector transfection, which alleviates these problems by using double stranded DNA alone to express the ribozyme. Direct DNA injection into skeletal muscle has been used in vaccine approaches for antigen expression. However, for other tissues, transfection efficiencies are greatly increased using cationic lipid/DNA complexes. Cationic lipid mediated DNA transfection yields a high transfection efficiency both *in vitro* and *in vivo* [34,35]. A Phase I clinical trial for the treatment of

melanomas has been completed using cationic lipids to deliver plasmid DNA. DNA encoding a foreign major histocompatibility antigen was complexed with cationic lipids and injected directly into the tumor. The patients' immune system recognizing the foreign antigen eliminated the tumors. Other protocols for this type of gene expression are in the review process for human clinical trials.

Permanent transfection is achieved with viral vectors that integrate into host DNA. The most widely used vector for these purposes has been the retroviral vectors [41–43]. As is the case for adenovirus, retroviral vector delivery uses replication incompetent viruses. The replication incompetent virions are produced by a packaging cell line transfected with the retrovirus genes necessary for virion production but lacking the ψ packaging sequence necessary for packing the viral genome into the virion. The ribozyme is cloned into a retroviral vector that contains only the long terminal repeats (LTRs) responsible for host DNA integration of the viral genome. The packaging cells produces virion particles that contain all the machinery for infection and replication but lack the viral genome. In its place is the ribozyme genome flanked by the LTRs and also containing a selectable marker gene to enrich for the transfected cell. These virions are then used to infect the target cell. Cells must be dividing for retroviral integration to occur. Retroviruses have a reasonable transfection efficiency, averaging 10%. Transfection is often performed *ex vivo* followed by selection of the permanently transformed cells. The cells are then returned to the patient. If the transfection frequency is reasonably high, cells may be returned to patients without selection. The disadvantages of retroviral vectors are low titers (between 10^5 and 10^6 PFUs/ml) and random integration into the host DNA.

Another vector under consideration is adeno-associated virus [44]. This expression vector is propagated in the same manner as the retroviral vectors, however, a helper virus such as adenovirus or one of the herpes viruses is also required. This vector offers the following advantages. i) The packaging cell lines produce similar titers to that of the retroviruses; however, unlike retroviruses, the adeno-associated virus can be concentrated. ii) Integration is specific for unmodified virus at human chromosome 19 and the only sequences required for integration are the flanking LTRs [45]. iii) Integration does not require that the target cells be dividing. This vector has just entered the gene therapy arena and extensive studies on long term expression, site specific integration, and potential activation by helper viruses are in progress.

Vectors applicable to ribozyme expression have been designed for protein expression, not RNA expression. For this reason, direct application of existing protein expression systems toward ribozyme delivery may not be straightforward. In all likelihood, the ribozyme may require a custom designed expression cassette to maximize the level of expression. In designing this vector system, several considerations must be taken into account.

In order for the ribozyme to be effective, it must either be in excess of the target RNA or colocalize with the target to increase the effective concentration [37]. If the ribozyme is transcribed from the same RNA polymerase as the target RNA, i.e., RNA polymerase II, the ribozyme promoter must be a stronger promoter than the target RNA, there must be multiple copies of the ribozyme expression cassette, or multiple ribozymes must be expressed under a single promoter. Ribozyme cleavage requires that the ribozyme form the hammerhead structure and the presence of additional ribozymes has the potential of yielding non-productive folding of the RNA. Incorporation of self processing ribozymes flanking either side of the therapeutic ribozymes would eliminate this possibility. The feasibility of this latter approach has been demonstrated [3].

An alternative would be to express the ribozyme under another promoter that had a higher transcription rate. Endogenous RNAs, such as snRNA and tRNA are transcribed by RNA Polymerase III, and ribosomal RNA is transcribed by RNA polymerase I. The endogenous RNA promoters are of interest because the abundance of these RNAs is greater than any one species of mRNA. A hairpin ribozyme shown to be effective against HIV was expressed from an RNA polymerase III tRNA promoter [9]. The transcription unit bearing the ribozyme was inserted into a retroviral vector and this vector was used to transfect primary human lymphocytes.

All ribozyme expression to date has been in *in vitro* systems. The approved protocol for HIV human gene therapy will be the first *in vivo* study. The goal of the protocol is a safety trial for the retroviral ribozyme expression vector. From these studies will also emerge preliminary results concerning the duration and expression level of ribozymes in human T cells *in vivo*. Second generation protocols will involve the transfection of human stem cells followed by autologous bone marrow transplantation. This approach will expand the number and types of immune cells capable of expressing the ribozyme.

There is a great promise in ribozyme technology because of the wide application toward the treatment of disease and medical disorders. Unlike the small molecule drugs that are effective in curing disease but often suffer from non-specific toxic side effects, ribozymes have exquisite specificity thus avoiding the toxic side effects. Ribozymes have the option of being delivered as a synthetic RNA or they can be expressed from an expression vector. The main limitation to this technology is the proper identification of a protein responsible for the disease state. In view of the rapid pace molecular cell biology is moving along with the progression of the human genome project, the significance of this limitation should be greatly reduced.

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